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# Effect of Recombinant Human Granulocyte Macrophage-Colony Stimulating Factor in Long-Term Marrow Cultures From Patients With Aplastic Anemia

**Guadalupe Martínez-Jaramillo,[1] Enrique Gómez-Morales,[2] and Hector Mayani[1]***

[1]Oncological Research Unit, Oncology Hospital, National Medical Center, Mexico City, Mexico
[2]Department of Hematology, "Bernardo Sepulveda" Hospital, National Medical Center, Mexico City, Mexico

**The hematopoietic system in patients with aplastic anemia (AA) shows both quantitative and qualitative deficiencies, i.e., reduced numbers of hematopoietic progenitor cells (HPC) and impaired HPC proliferation in long-term marrow cultures (LTMC). Since recombinant human granulocyte macrophage-colony stimulating factor (rhGM-CSF) has been shown to be a potent stimulator of normal hematopoiesis, both in vivo and in vitro, in the present study we wanted to assess the possibility of stimulating hematopoiesis in LTMC from 17 patients with AA, by weekly addition of rhGM-CSF (10 ng/ml). In LTMC from 11 patients (group of responders), rhGM-CSF induced a significant increase (4.8-fold, compared with untreated cultures) in the levels of myeloid progenitor cells; in contrast, in six patients (group of nonresponders), myeloid progenitors were refractory to this cytokine. In the group of responders, rhGM-CSF also induced a pronounced increment in the levels of nonadherent and adherent cells (5.99- and 5.18-fold, respectively, compared with untreated cultures). Among the different myelopoietic lineages, rhGM-CSF preferentially stimulated the macrophagic lineage; this was evident both at the progenitor and mature cell levels. Interestingly, the effect of rhGM-CSF in LTMC from AA patients was only transient. Indeed, the effects mentioned above were observed only during the first three weeks of culture; afterwards, myeloid progenitor and nonadherent cell levels in treated cultures declined, practically reaching the levels observed in untreated cultures. At the moment, we do not know whether this transient stimulatory effect is due to the production of inhibitory cytokines, by macrophages generated in response to rhGM-CSF, or to the exhaustion of the HPC pool in AA cultures. In all 17 patients, rhGM-CSF had no effect on the kinetics of erythroid or multipotent progenitor cells. These results are in keeping with clinical studies in which it has been observed that most AA patients treated with rhGM-CSF show increments in circulating monocytes and granulocytes, as well as in bone marrow cellularity. However, little or no effect is observed on erythropoiesis. The actual mechanisms involved in the in vitro effects of rhGM-CSF on myeloid progenitor cells from AA bone marrow are still not completely understood. Future studies on this issue should be encouraged, since they may help to understand the in vivo (clinical) effects of this cytokine. Am. J. Hematol. 61:107–114, 1999.** 

**Key words: aplastic anemia; bone marrow; hematopoiesis; long-term marrow cultures; macrophages; progenitor cells; rhGM-CSF**

## INTRODUCTION

Aplastic anemia (AA) is characterized by deficiencies in the hematopoietic system, which lead to the poor production of mature blood cells. Thus, patients with AA show pancytopenia and a hypocellular bone marrow [1]. The pathogenesis of this disease, however, is still not completely understood and alterations both in the stem/



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*Correspondence to: Hector Mayani, Ph.D., Oncological Research Unit, Oncology Hospital, National Medical Center, IMSS, Av. Cuauhtemoc 330, Col. Doctores Mexico, D.F. 06720, Mexico

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progenitor cell compartment and in the hematopoietic microenvironment have been documented [2–5].

Several studies have demonstrated that patients with AA show extremely reduced levels of marrow CD34+ cells, as well as of hematopoietic progenitor cells (HPC) capable of forming colonies in semisolid cultures [2,3,6–7]. Interestingly, not only quantitative but also qualitative alterations in hematopoiesis have been observed. Indeed, it has been recently reported that the frequency of apoptosis is significantly increased in HPC from AA patients, as compared with normal subjects [8], and in vitro studies indicate that the proliferative capacity of HPC from patients with AA is greatly deficient [9–12]. Different groups, including our own, have clearly shown that when bone marrow cells from AA patients are cultured in Dexter-type long-term marrow cultures (LTMC), HPC growth is markedly impaired and these cells reach undetectable levels after only a few weeks in culture [10,11,13,14]. This was observed even in LTMC from patients that achieved complete remission after immunosuppressive therapy [14].

Human hematopoiesis in LTMC can be modulated by using stimulatory and inhibitory hematopoietic cytokines [15–18]. In this regard, it is of particular relevance the use of recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF), one of the main stimulators—both in vivo and in vitro—of granulocyte and macrophage production [19,20]. Studies by Haas et al. [21], Coutinho et al. [22], and Charbord et al. [23] have demonstrated that treatment of normal marrow cells with rhGM-CSF, prior or during LTMC, resulted in significant increments in the numbers of both progenitor and mature myeloid cells, thus indicating that addition of rhGM-CSF to LTMC from normal bone marrow stimulates hematopoiesis.

Based on all of the above observations, we carried out the present study. Our main objective was to assess the possibility of stimulating the growth of AA-derived HPC and their progeny in LTMC, by weekly addition of rhGM-CSF. Accordingly, the kinetics of total nucleated cells and HPC, including myeloid, erythroid and multipotent progenitors, were followed, both in untreated and treated cultures, during a five-week culture period.

## MATERIALS AND METHODS

### Cell Collection

Bone marrow (BM) cells, collected according to institutional guidelines, were obtained from 17 patients with severe AA. Diagnosis was established by BM biopsy and peripheral blood count criteria, according to the International Agranulocytosis and Aplastic Anemia Study Group [24]. By the time of this study, all the patients had been subjected to immunosuppressive therapy (antilymphocyte globulin and/or cyclosporin A). Five of them achieved complete remission; nine of them showed partial response; whereas three of them did not respond to the treatment.

### Cell Processing

Buffy coat cells from AA marrow were obtained by centrifugation (400 g for 7 min) and low-density mononuclear cells (<1.077 g/ml) were isolated using Ficoll-Paque Plus (Pharmacia Biotech, Uppsala, Sweden). Cells were then resuspended in Iscove's modified Dulbecco's medium (IMDM) supplemented with 2% fetal bovine serum (FBS; StemCell Technologies Inc. [STI], Vancouver, BC, Canada). Total numbers of nucleated and viable cells were determined with a hemocytometer, using Turck's solution and trypan blue stain, respectively.

### Hematopoietic Colony Assays

Hematopoietic progenitor cells were assayed in methylcellulose-based semisolid cultures (STI). The culture medium consisted of 0.9% methylcellulose, 30% FBS, 1% bovine serum albumin, $10^{-4}$ M 2-mercaptoethanol, 2 mM L-glutamine, 50 ng/ml recombinant human (rh) stem cell factor (SCF), 10 ng/ml rh Interleukin-3 (IL-3), 10 ng/ml rh granulocyte-macrophage colony-stimulating factor (GM-CSF), and 3 U/ml rh erythropoietin (EPO). Low-density mononuclear cells were plated at a final concentration of $5 \times 10^4$ cells/ml and the cultures were incubated at 37°C in an atmosphere of 5% $CO_2$ in air. Nonadherent cells from LTMC were also cultured in this manner; however, the plating cell concentration varied from $5 \times 10^4$ to $1 \times 10^4$, depending on the cell number recovered from the cultures. After 14–17 days of culture, colonies were scored in the same dish using an inverted microscope. Hematopoietic colonies were classified as follows: CFU-MIX, colonies containing both erythroid and myeloid cells; CFU-E, erythroid clusters of 20–50 hemoglobinized cells; BFU-E, erythroid colonies of more than 50 hemoglobinized cells grouped in one or several clusters. Myeloid colonies comprised the identifiable subpopulations of pure granulocytic colonies (CFU-G), pure macrophagic colonies (CFU-M), and colonies containing both granulocytes and macrophages (CFU-GM).

### Long-Term Marrow Cultures

LTMC were established as previously described [25]. Low-density mononuclear cells were resuspended in LTMC medium (STI) at a final concentration of $3 \times 10^6$ cells per milliliter. The LTMC medium composition is as follows: alpha medium supplemented with 12.5% horse serum, 12.5% FBS, 0.2 mM inositol, 20 μM folic acid, $10^{-4}$ M 2-mercaptoethanol, 2 mM L-glutamine, and freshly dissolved hydrocortisone to yield a final concentration of $10^{-6}$ M. The cell suspension was loaded into 24-well plates (1 ml/well) and incubated at 37°C in an

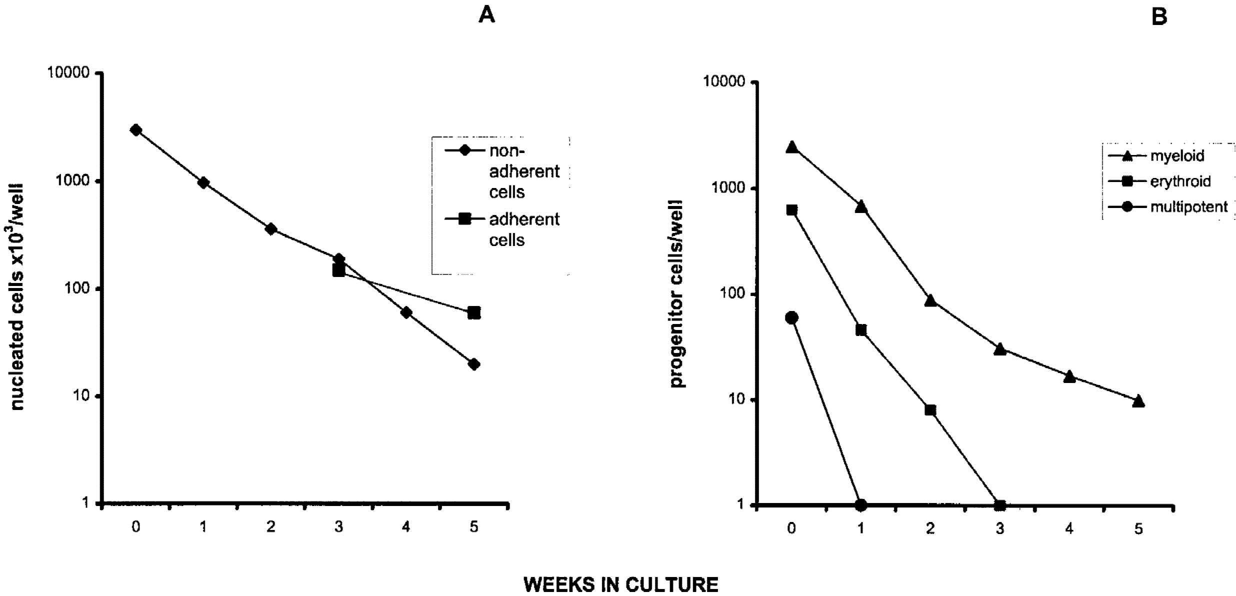


**Fig. 1. Kinetics of total nucleated (A) and progenitor (B) cells, throughout a five-week culture period, in untreated long-term marrow cultures from patients with aplastic anemia. Results represent median values of the cultures established from 17 patients.**

atmosphere of 5% $CO_2$ in air. After three days cultures were transferred to a different incubator and maintained at 33°C. Four days later (seven days after initiation of the culture) one-half of the supernatant and nonadherent cells were removed from the wells and replaced with fresh culture medium. The cultures were processed in this manner at weekly intervals. The nonadherent cells, obtained weekly during medium change, were counted, morphologically analyzed, and assayed for hematopoietic progenitors. At weeks 3 and 5 one of a number of parallel cultures was sacrificed for evaluation of the adherent cells. These were detached with a cell scraper after trypsinization (i.e., 0.25% trypsin containing 0.1 mM EDTA was added and the cultures were incubated at 37°C for 10 min; the action of trypsin was stopped by adding one-half volume of FBS). The cells were then resuspended in IMDM with 2% FBS and processed in the same way as the nonadherent cells.

### Addition of rhGM-CSF

From each particular bone marrow sample, four to six LTMC were established as described above. One-half of them were treated with rhGM-CSF (molgramostim; Novartis/Schering-Plough, Basel, Switzerland), which was added weekly, during medium change, at 10 ng/ml. Evaluation of rhGM-CSF-treated cultures was done as for untreated cultures.

### Statistics

Statistical analysis was performed by using the Mann-Whitney U test.

## RESULTS

### Kinetics of Nucleated and HPC in Untreated LTMC From AA Patients

The kinetics of nucleated cells—both in the nonadherent and adherent fractions–as well as the kinetics of myeloid, erythroid, and multipotent progenitors in LTMC from AA patients, have been thoroughly described in our previous report [14]. In keeping with such a report, in the present study all of these cell populations showed very low levels throughout the five-week culture period (Figure 1). Indeed, such levels were significantly reduced as compared with those in normal marrow-derived LTMC (for comparison see references 14 and 25). In all the cases, total nonadherent cell levels were reduced five- to fourteenfold, as compared with normal marrow LTMC, and adherent cells were reduced two- to sixfold. In terms of the different types of HPC, myeloid progenitor levels in AA cultures were reduced three- to sixteenfold, as compared with normal LTMC. Erythroid progenitors were reduced four- to twelvefold and reached undetectable levels significantly faster than in normal marrow cultures (week 3 in AA LTMC vs. week 6 in normal LTMC). Finally, multipotent progenitors were undetectable in AA LTMC (Figure 1B), whereas they were sustained for three to four weeks in normal cultures.

### Effect of rhGM-CSF on HPC Levels in LTMC From AA Patients

The levels of HPC in rhGM-CSF treated cultures were followed during a five-week culture period. Recombinant human GM-CSF had no significant effect on the levels of

**TABLE I. Effect of rhGM-CSF on the Levels of Myeloid Progenitor Cells in LTMC From Patients with Aplastic Anemia***

| Day | Responders ($n = 11$) | Nonresponders ($n = 6$) |
|---|---|---|
| 0 | 1.00 | 1.00 |
| 7 | 4.81 (2.72–16.10)[a] | 0.97 (0.88–1.61) |
| 14 | 3.93 (2.46–13.27)[a] | 1.06 (0.81–1.66) |
| 21 | 2.67 (1.19–7.80)[a] | 0.83 (0.73–1.42) |
| 28 | 1.29 (0.90–3.22) | 0.91 (0.37–1.17) |
| 35 | 0.46 (0.19–1.77) | 0.79 (0.41–1.23) |

*Results are expressed as median (range) of the fold-increase as compared with untreated cultures.
[a]Significantly different ($p < 0.05$) from untreated cultures.

erythroid progenitor cells. In fact, whenever these cells were detected in LTMC, their levels in treated cultures were 79–132% of the levels found in untreated cultures (not shown). As shown in Figure 1B, multipotent progenitor cells were not detected in untreated LTMC throughout the five-week culture period. This was also the case in rhGM-CSF-treated cultures (not shown). In contrast, in the majority of the AA patients included in this study, rhGM-CSF had significant effects on the levels of myeloid progenitors.

In 11 out of 17 patients (65%; group of responders), rhGM-CSF induced a significant increase in the levels of myeloid progenitors (Table I). Patients were considered to be responders to rhGM-CSF if their HPC levels in rhGM-CSF treated LTMC were increased more than twofold, as compared with untreated cultures. The maximum increase was observed during the first week of culture (median increase = 4.8-fold, as compared with untreated cultures). In contrast, in 6 of 17 patients (35%; group of nonresponders), rhGM-CSF had no effect on the levels of myeloid progenitors (Table I). Interestingly, the effect of rhGM-CSF observed in the group of responders was only transient. That is to say, after the initial increment observed during the first three weeks of culture, myeloid progenitor levels decreased—in spite of the continuous addition of rhGM-CSF—and by week 4 their levels were similar to those in untreated cultures. Furthermore, by week 5, myeloid progenitor levels in most LTMC from this group of patients were lower than the levels found in untreated cultures (Table I).

When analyzing the relative proportion of the different subtypes of myeloid progenitors, both in treated and untreated cultures, we found that on day 0 CFU-G were the most numerous (75%), followed by CFU-M (20%), and CFU-GM (5%) (Table II). These proportions were similar to those reported for normal bone marrow [25]. After seven days of culture, the proportion of the different types of myeloid progenitor cells in untreated cultures, as well as in the group of nonresponders, was similar to that on day 0; in contrast, a significant increase in the proportion of CFU-M was observed in the group of responders (Table II). Moreover, on day 28 CFU-M was the most numerous progenitor cell type in cultures of this latter group. In terms of absolute numbers, on day 7 of culture CFU-M levels were increased thirteenfold, as compared with untreated and nonresponders' LTMC, whereas CFU-G levels were increased only threefold. On day 28, CFU-M numbers were threefold higher in responders' LTMC than in untreated/nonresponders' cultures. In contrast, CFU-G levels were higher in untreated/nonresponders' LTMC. These results indicate that rhGM-CSF preferentially stimulated the growth of macrophagic progenitors.

### Effect of rhGM-CSF on Total Nucleated Cells in LTMC From AA Patients

The levels of total nonadherent cells were also quantitated throughout the five-week culture period. In keeping with the results observed in terms of HPC, those patients who responded to rhGM-CSF by increasing myeloid progenitor cell levels also showed a significant increase in the number of nucleated cells. Indeed, in the group of responders, total nonadherent cell levels were increased up to sixfold, as compared with untreated cultures. However, whereas the peak in myeloid progenitor cells was observed on day 7 (Table I), total nonadherent cells reached their peak on day 21 (Table III). Interestingly, on day 35 nonadherent cells in LTMC from this group of patients decreased, almost reaching the levels observed in untreated cultures. In the nonresponders group, nonadherent cell levels were always similar to those observed in untreated cultures.

On days 21 and 35, total numbers of adherent cells were determined. LTMC from responders patients showed a 3.28- and 5.18-fold increase on weeks 3 and 5, respectively (Table IV; Figure 2). In LTMC from nonresponders the levels of adherent cells were also increased (1.69- and 2.48-fold, on weeks 3 and 5, respectively), although not to the same extent as in LTMC from the former group. Morphological analysis under the inverted microscope (Figure 2) and from slides stained with Wright-Giemsa, indicated that most of the adherent cells present in LTMC treated with rhGM-CSF consisted of macrophages.

## DISCUSSION

Previous studies by different groups have demonstrated that the hematopoietic system of patients with AA shows both quantitative and qualitative deficiencies, i.e., reduced numbers of HPC and impaired HPC proliferation in LTMC [2,3,6–7,9–14]. Our own group has shown that such deficiencies are present even in AA patients that have achieved complete remission after immunosuppressive therapy [14]. Since rhGM-CSF has been shown to be a potent stimulator of normal hematopoiesis, both in vivo and in vitro, in the present study we wanted to

**TABLE II. Relative Proportion of Myeloid Progenitor Cells in rhGM-CSF Treated LTMC From Patients With Aplastic Anemia***

| | Responders ($n = 11$) | | | Nonresponders ($n = 6$) | | |
|---|---|---|---|---|---|---|
| Day | CFU-G | CFU-M | CFU-GM | CFU-G | CFU-M | CFU-GM |
| 0 | 76 (48–100) | 20 (0–48) | 2 (0–26) | 75 (41–93) | 18 (7–38) | 5 (0–20) |
| 7 | 53 (23–81)[a] | 37 (17–59)[a] | 11 (0–31) | 80 (63–98) | 13 (2–26) | 7 (0–14) |
| 28 | 24 (13–39)[a] | 76 (47–91)[a] | 0 (0–7) | 60 (46–81) | 39 (27–46) | 0 (0–11) |

*Results (%) represent median (range) of the total number of myeloid progenitors.
[a]Significantly different ($p < 0.05$) from the results in the group of nonresponders.

**TABLE III. Effect of rhGM-CSF on the Levels of Nonadherent Cells in LTMC From Patients With Aplastic Anemia***

| Day | Responders ($n = 11$) | Nonresponders ($n = 6$) |
|---|---|---|
| 0 | 1.00 | 1.00 |
| 7 | 1.85 (1.06–2.80) | 1.06 (0.66–1.27) |
| 14 | 3.87 (1.71–5.25)[a] | 1.26 (0.90–1.53) |
| 21 | 5.99 (2.30–9.36)[a] | 1.57 (0.81–2.61) |
| 28 | 4.07 (1.70–6.46)[a] | 1.42 (0.71–1.90) |
| 35 | 1.62 (0.83–3.75) | 1.13 (0.88–1.63) |

*Results are expressed as median (range) of the fold-increase as compared with untreated cultures.
[a]Significantly different ($p < 0.05$) from untreated cultures.

**TABLE IV. Effect of rhGM-CSF on the Levels of Adherent Cells in LTMC From Patients With Aplastic Anemia***

| Day | Responders ($n = 11$) | Nonresponders ($n = 6$) |
|---|---|---|
| 21 | 3.28 (1.47–4.19)[a] | 1.69 (0.87–2.19) |
| 35 | 5.18 (3.00–6.68)[a] | 2.48 (1.32–3.37)[a] |

*Results are expressed as median (range) of the fold-increase as compared with untreated cultures.
[a]Significantly different ($p < 0.05$) from untreated cultures.

assess the possibility of stimulating human hematopoiesis in LTMC from AA patients by weekly addition of rhGM-CSF.

In 11 of 17 AA patients included in this study (65%), addition of rhGM-CSF (10 ng/ml) to LTMC resulted in a significant increase in the levels of myeloid progenitor cells. In contrast, in six of 17 patients (35%), myeloid progenitors were refractory to the addition of rhGM-CSF. This latter observation differs from the results reported by Coutinho et al., who found that, in LTMC from normal bone marrow, rhGM-CSF (10 ng/ml) induced a significant increment in the levels of myeloid progenitors in 100% of the cases [22]. Although the actual reason for such a lack of response to rhGM-CSF is not known, different possibilities could be suggested: (a) abnormal expression of GM-CSF receptors (GM-CSFR), (b) impairments in the GM-CSFR-related signal transduction pathways, or (c) alterations at the level of transcription factors.

Those patients in whom myeloid progenitor levels were raised in response to rhGM-CSF also showed a significant increase in the levels of nucleated cells, both nonadherent and adherent. Interestingly, whereas myeloid progenitors reached their maximum levels on day 7 of culture, nonadherent and adherent cells reached their peaks on days 21 and 35, respectively. This suggests that the increments observed in nucleated cell levels were the result of the stimulated proliferation of myeloid progenitors. Our results are in keeping with those of Coutinho et al., since in their study, rhGM-CSF-stimulated myeloid progenitors also reached their peak two weeks before nonadherent cell levels did [22]. However, in contrast to the present study, in the study by Coutinho and colleagues myeloid progenitors from normal marrow reached their peak by week 3 of culture. Thus, it is possible that in those AA patients who responded to rhGM-CSF, myeloid progenitors possess an increased sensitivity to this cytokine, as compared with normal progenitors.

In the culture system reported here, rhGM-CSF preferentially stimulated the macrophagic lineage of hematopoiesis. This was observed both at the progenitor and mature cell levels. Previous reports by Metcalf et al. [26] and Mayani et al. [27], using semisolid cultures of normal human marrow, indicated that although rhGM-CSF is capable of stimulating all three types of myeloid progenitors (i.e., CFU-G, CFU-M, and CFU-GM), it preferentially stimulates the growth of CFU-M. Our results indicate that this preferential effect is also present in AA LTMC. Indeed, in LTMC from the group of responders, CFU-M levels were increased up to thirteenfold, as compared with untreated and nonresponder LTMC. In contrast, CFU-G numbers were only increased threefold.

Using LTMC from normal human bone marrow, Charbord and colleagues [23] demonstrated that addition of rhGM-CSF (10 or 50 ng/ml) induced the generation of macrophages, which constituted the vast majority of the cells present in the adherent layer. A similar observation was made in the present study. In LTMC from AA patients who responded to rhGM-CSF macrophage levels were increased more than eightfold, as compared with untreated LTMC. This, of course, could be explained by the increment observed in CFU-M levels. Interestingly, rhGM-CSF also induced a significant increase in the levels of adherent macrophages in cultures of AA patients that did not show any increase in myeloid progenitor

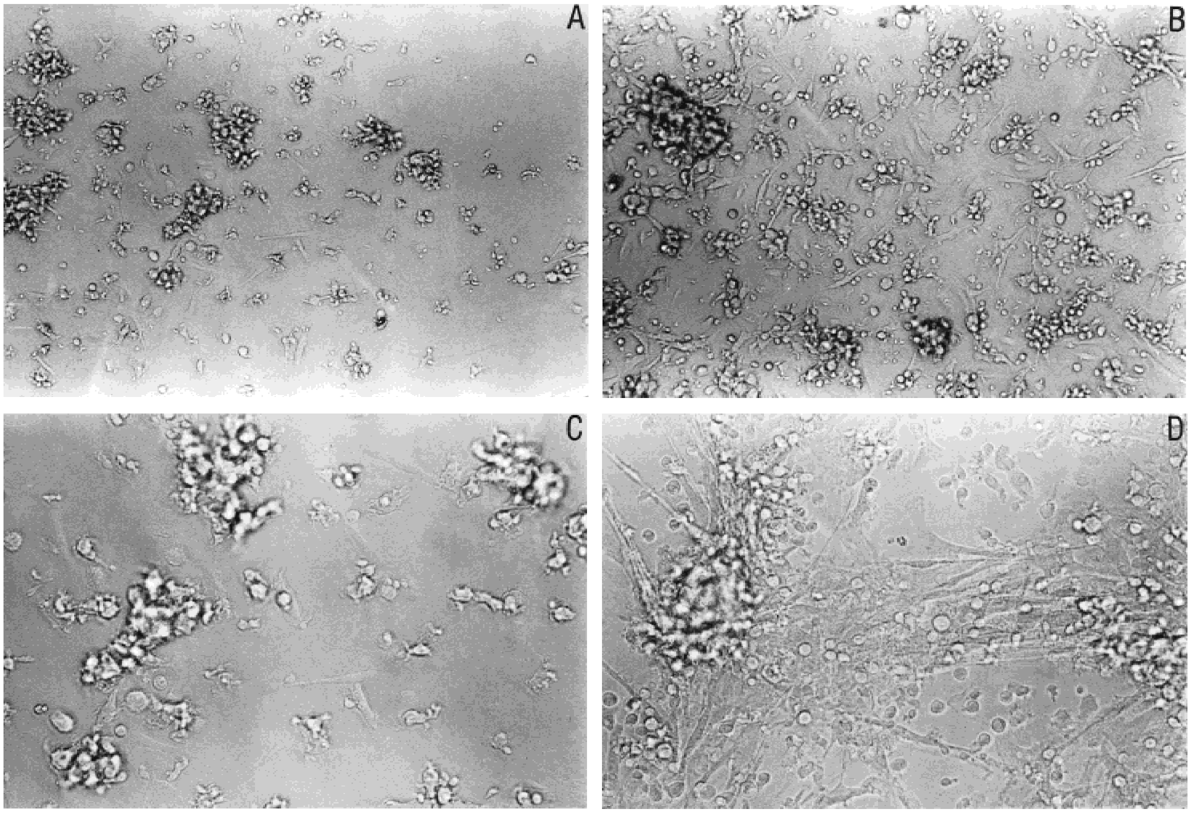


**Fig. 2. Appearance under the inverted microscope of a representative (week 4) long-term marrow culture from a responder patient with aplastic anemia. A and C, untreated culture; B and D, culture treated with rhGM-CSF (10 ng/ml, added weekly during culture medium change). Note that the number of both stromal and hematopoietic cells are significantly increased in the rhGM-CSF-treated culture. A and B, ×100; C and D, ×200.**

cells. The actual reason for this observation is not known; however, two possible explanations could be suggested. First, although in that group of patients rhGM-CSF did not induce any increment in the numbers of CFU-M, it may be acting on these cells by stimulating their capacity for cell division, so that individual CFU-M were able to produce larger numbers of mature macrophages. A second possibility would be that rhGM-CSF may not be acting on CFU-M but on relatively mature monocytes/macrophages, inducing their proliferation. The results of the present study do not allow us to distinguish between these possibilities.

One of the most striking observations of the present study was the fact that the effect of rhGM-CSF on AA-derived myeloid progenitors was a transient one. Indeed, in spite of the weekly addition of this cytokine to AA LTMC, myeloid progenitors showed increased levels, as compared with untreated cultures, only during the first three weeks. By weeks 4 and 5 their levels were similar and lower, respectively, than those in untreated cultures. A possible explanation for this observation is that the elevated number of macrophages, generated from week 3 in response to rhGM-CSF, produced one or more inhibitory cytokines (e.g., tumor necrosis factor-$\alpha$), which, in turn, acted on HPC by inhibiting their growth. This would be similar to what has been observed in normal marrow LTMC treated with rhMacrophage-CSF [28]. A second possibility is that the in vitro HPC pool, dramatically reduced in AA patients, was rapidly exhausted by the action of rhGM-CSF; thus, after the first three weeks of culture HPC levels were almost depleted. Furthermore, rhGM-CSF could also be acting on more mature precursors, which would become exhausted unless replenished by an influx from immature progenitors. Either of the two possibilities mentioned above could explain the fact that by week 5 of culture, myeloid progenitor levels in the group of responders were below the levels observed in untreated cultures.

Interestingly, it seems that the transient effect of rhGM-CSF on HPC growth is not exclusive to AA LTMC. In the study by Coutinho et al. [22], rhGM-CSF also induced a transient stimulation of the levels of myeloid progenitors in normal LTMC. Such a period of stimulation, however, was longer than the one observed in the present study (five weeks vs. three weeks). Moreover, no significant inhibition of HPC growth was observed after the period of stimulation. In the study by Charbord and colleagues [23], on the other hand, addi-

tion of 50 ng/ml rhGM-CSF to normal LTMC resulted in a dramatic inhibition of HPC levels. The authors suggested that the increased generation and accumulation of macrophages mediated this inhibition. Similar observations—i.e., increased generation of macrophages and inhibition of HPC growth—were made in the present study, although the rhGM-CSF concentration used here was lower.

It is noteworthy that in the present study, rhGM-CSF had no significant effect on the growth of erythroid progenitor cells. This may be due to the fact that (a) we did not supplement the LTMC with exogenous erythropoietin, and that (b) rhGM-CSF, by itself, is not an erythropoietic factor [19,20]. These results correlate with different studies on the in vivo effects of rhGM-CSF in patients with AA. In such studies, treatment of AA patients with this cytokine resulted in significant increments in the levels of circulating granulocytes and monocytes, as well as in bone marrow cellularity and myeloid progenitor cells. In contrast, the effects on erythropoiesis were mild and observed only in some patients [29,30]. Thus, indicating that other cytokines, apart from rhGM-CSF, are necessary for the stimulation of erythropoiesis in AA patients. Also in relation to in vivo studies, Nissen and colleagues have shown that in some AA patients, rhGM-CSF is unable to stimulate the production of granulocytes and monocytes [31]. This correlates with the observation made in the present study that in some AA patients, myeloid progenitor cells are refractory to the in vitro effects of this cytokine.

It has to be kept in mind that all the patients included in this study had been previously treated with immunosuppressive therapy, thus the results shown here may not be representative of unselected, newly diagnosed patients. It would be interesting to see if HPC from untreated patients respond to rhGM-CSF in the same way as HPC from treated patients did. Interestingly, we did not find any correlation between the in vitro response to rhGM-CSF and the in vivo response of patients to the immunosuppressive therapy (not shown).

To our knowledge, this is the first full report on the characterization of the effect of rhGM-CSF, added as a single factor, in LTMC from AA patients. In a previous study, Gibson and colleagues analyzed the effect of rhGM-CSF (100 ng/ml), in combination with rh interleukin-3 (rhIL-3; 100 ng/ml) and rh stem cell factor (rhSCF; 100 ng/ml) in AA LTMC [32]. They found that the combination of rhGM-CSF plus rhIL-3 was able to stimulate nonadherent cells and myeloid progenitor levels up to 4.9- and 4.8-fold, respectively, as compared with untreated cultures. In their study, the peak in myeloid progenitor cell levels was achieved on week 4 of culture. Interestingly, hematopoiesis was not improved by the addition of rhSCF to the two-factor combination. Erythroid and multipotent progenitor cell levels were not significantly influenced by addition of the two- or three-factor combination [32]. The fact that in the study by Gibson et al. rhGM-CSF was added together with other factors, and that the cytokine concentration used by them was tenfold higher than the one used in the present study, makes it difficult to analyze both studies in a comparative way. However, it is clear that a similar trend (i.e., stimulation of myeloid progenitor and mature cells, reaching maximum levels on particular days of culture, and lack of an effect on erythroid or multipotent progenitor cell levels) was observed in both studies.

The actual mechanisms involved in the in vitro effects of rhGM-CSF on human hematopoiesis, both from normal and AA bone marrow, are still not completely understood. Future studies on this issue should be encouraged, since they may help to understand the in vivo (clinical) effects of this cytokine, which has been widely used in the treatment of several hematological diseases, besides AA.

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